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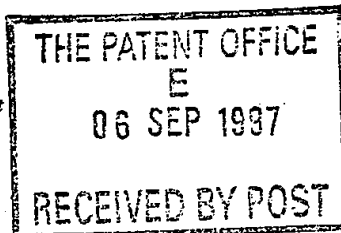
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1. Your reference

HCM/MJL/C1157/M

2. Patent application number

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9718872.6

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Medical Research Council
20 Park Crescent, London
WIN 4AL

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom 596007001

4. Title of the invention

Improvements in or Relating to
Expression of Immunogenic Substances

5. Name of your agent (if you have one)

Keith W Nash & Co

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

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Patents ADP number (if you know it)

1206001

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Country

Priority application number
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Date of filing
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Number of earlier application

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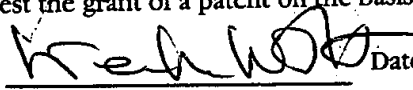
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C1157.00/M

Title: Improvements in or Relating to Expression of Immunogenic Substances

Field of the Invention

This invention relates, *inter alia*, to methods of regulating the expression of nucleic acid sequences in eukaryotic cells and, in particular, to a method of regulating the expression of immunogenic polypeptides, and to a method of altering the sensitivity of a leukocyte to a target antigen.

Background of the Invention

Unlike antibody molecules, T cells can migrate actively and efficiently through microvascular walls, allowing them to penetrate the core of a solid tumor before they exert their cytolytic function. *Ex vivo* expansion and re-infusion of autologous tumorreactive T cells is being explored as an experimental approach to cancer therapy. However, circulating T cells from peripheral blood lack specificity for tumor antigens (1) and it is often impractical or impossible to obtain sufficient numbers of tumor infiltrating lymphocytes (2). To overcome these problems new approaches have been developed whereby antibody specificity can be combined with the efficient trafficking properties and effector functions of T cells. Several reports have demonstrated the feasibility of transfecting cultured T cells with genes encoding chimeric receptors in which single-chain antibody domains (scFv) are linked to different signalling portions of the TCR/CD3/ ζ complex as a means to target T cells towards native antigens (3-6). However it is apparent that the long-term clinical success of this "T-body" approach could depend on the development of solutions to a number of problems.

Perhaps the most significant concern is that since few "cancer antigens" are truly tumor-specific (7), successful therapy with tumor-reactive T-bodies could be associated with significant collateral damage to normal tissues expressing low levels of the targeted

antigen. It will therefore be desirable to develop strategies by which T cells can be rendered temporarily or permanently anergic to their target antigen, or differentially sensitive to different surface densities of the antigen on target cell membranes.

Although different strategies have been developed for regulating transgene expression in eukaryotic cells (8), the tetracycline-regulatable system (TRS) avoids the problems related to many of these systems by offering substantial regulation of transgene expression in response to concentrations of tetracycline that cause little or no toxicity in mammalian cells (9, 10).

Miller & Whelan (1997 *Hum. Gene Therapy* **8**, 803-815) have recently reviewed progress towards the development of regulatable vectors for gene therapy. Among the vectors described are those using the TRS.

In the TRS originally described by Gossen & Bujard (1992 *Proc. Natl. Acad. Sci. USA* **89**, 5547-5551), the tetracycline repressor protein was fused to the Herpes Simplex Virus (HSV) VP16-activating domain, to create a chimeric tetracycline-repressible transactivating (tTA) polypeptide, which binds to DNA comprising the tet operator sequence, causing transcriptional activation of coding sequences downstream of the operator. The presence of tetracycline or analogues thereof (such as doxycycline, anhydrotetracycline, minocycline and oxytetracycline) inhibits this transcriptional activation, as these compounds bind to the tTA polypeptide, altering its conformation and prevent its binding to the tet operator sequence.

Variants of the original TRS have now been described (WO 96/01313) in which a mutant form of the tet repressor protein binds to DNA in the presence, but not in the absence, of tetracycline or its analogues. Thus, in these systems, expression of a tet operator-linked gene is positively regulated in the presence of tetracycline or its analogues.

However, there are a number of difficulties and/or uncertainties relating to the use of the TRS. For example, Cooke *et al*, (1997 *J. Gen. Virol.* **78**, 381-392) found that the TRS

could not be used to regulate expression of the *nef* gene in the human T cell line Jurkat E6-1. Further, the HSV VP16 domain is associated with a toxic "squenching" effect, such that high levels of regulation have not previously been obtainable. A recent review (Miller & Whelan 1997 Hum. Gene Ther. 8, 803-815) has stated that the TRS "may not be applicable to all cell lines" (citing the work of Ackland-Berglund & Leib 1995 BioTechniques 18, 196-200; and Howé *et al.*, 1995 J. Biol. Chem. 270, 14168-14174).

Summary of the Invention

In a first aspect the invention provides a method of regulating the expression of a nucleic acid sequence encoding a heterologous polypeptide in a leukocyte, comprising introducing into the leukocyte the nucleic acid coding sequence operably-linked to a tetracycline-operator sequence, and a sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide; and altering the concentration of tetracycline (or analogues thereof) to which the leukocyte is exposed, so as to regulate expression of the coding sequence.

The expression-regulating polypeptide may be such that, when bound to DNA, it causes positive regulation (or up-regulation) of a coding sequence, or it may be a polypeptide which causes negative regulation (or down-regulation) of a coding sequence. Examples of both types of protein are well-known to those skilled in the art. For example, in the absence of tetracycline the wild-type bacterial tet repressor protein causes negative regulation of bacterial tetracycline-resistance genes: tetracycline binds to the repressor protein and prevents it from binding to the tet operator DNA sequence, thus allowing expression of the resistance genes.

Conversely, a chimeric polypeptide comprising part of the tet repressor and the HSV VP16 transactivating domain causes positive regulation (transcriptional activation) of coding sequences. Other transactivating domains are known to those skilled in the art (e.g. amino acid residues 753-881 of GAL4; amino acid residues 399-499 of CTF/NF1; and those from ITF1 or ITF2), and these may conceivably be used to form a chimeric tetracycline-sensitive polypeptide.

In addition to the chimeric polypeptides described in the prior art (e.g. Gossen & Bujard 1992, cited above) which comprise a portion of the wild type tet repressor protein, other polypeptides are known (disclosed in WO 96/01313) which comprise a mutated form of the tet repressor protein, which binds to the tet operator sequence in the presence, but not in the absence, of tetracycline.

Accordingly the method of the invention is such that in some embodiments the presence of tetracycline will serve to inhibit expression of the tetracycline operator (tet O) - linked coding sequence, whilst in other embodiments the presence of tetracycline will serve to enhance expression of the tet O-linked sequence.

Those skilled in the art will appreciate that a number of analogues of tetracycline are known, which can readily be substituted for tetracycline in the method of the invention. Indeed, certain analogues may actually be preferred to tetracycline, as they may have higher binding affinities for the tetracycline-sensitive polypeptide. Preferred analogues are doxycycline and anhydrotetracycline. Other analogues include minocycline, oxytetracycline, chlorotetracycline, epioxytetracycline and cyanotetracycline. Other analogues of tetracycline are described by Hlavka & Boothe ("The Tetracyclines" in "Handbook of Experimental Pharmacology 78, Blackwood *et al*, (eds.) Springer Verlag 1985).

The tet operator (tet O) sequence is now well-known to those skilled in the art. For a review, the reader is referred to Hillen & Wissmann (1989) in Protein-Nucleic Acid Interaction. "Topics in Molecular and Structural Biology", eds. Saenger, W D Heinemann. U. (Macmillan, London), Vol. 10, pp 143-162. Typically the nucleic acid sequence encoding the heterologous polypeptide will be placed downstream of a plurality of tet O sequences: generally 5 to 10 such tet O sequences are used, in direct repeats.

Distance between the operators and position +1 of PhLMV is 19 bp (in PhLMV-1). Conveniently, the tet O sequences will be fused substantially adjacent (i.e. within 100bp, preferably within 50 bp) to a "minimal" (i.e. enhancerless) eukaryotic promoter (such as the minimal CMV immediate early promoter, described previously [Gossen & Bujard 1992

Proc. Natl. Acad. Sci. USA **89**, 5547]), such that binding of a transactivating tetracycline-sensitive polypeptide to the tet O sequence will cause enhanced expression of the tet O-linked coding sequence.

The sequence encoding the heterologous polypeptide, and the sequence encoding the tetracycline-sensitive expression-regulating polypeptide may, if desired, be introduced into the leukocyte on separate nucleic acid constructs. More preferably however, both sequences will be introduced into the leukocyte on a single nucleic acid construct. Constructs suitable for this task have been disclosed previously (e.g. Baron *et al*, 1995 Nucl. Acids Res. **23**, 3605-3606; Schultze *et al*, 1996 Nature Biotechnology **14**, 499-503). In some embodiments, it will be particularly desirable if the expression of the tetracycline-sensitive expression-regulating polypeptide is also tet O-linked, allowing for a positive feedback loop in the absence of tetracycline (see Shockett *et al*, 1995 Proc. Natl. Acad. Sci. USA **92**, 6522-6526; and Hofmann *et al*, 1996 Proc. Natl. Acad. Sci. USA **93**, 5185-5190). A particularly preferred construct, found to allow for very high levels of regulation of expression of tet O-linked sequences is disclosed in the co-pending UK patent application filed on even date herewith (under reference C1164/M), a copy of which is appended hereto.

The method of the invention finds application in a number of fields, but particularly in the field of gene therapy. Thus, whilst the method of the invention may be useful in regulating the expression of any polypeptide heterologous to (i.e. not naturally expressed in) a leukocyte, it is desirably applied to the regulation of the expression of one or more therapeutic polypeptides. Those skilled in the art are familiar with the wide range of polypeptides which have potential usefulness when expressed as therapeutic polypeptides in patients by means of gene therapy techniques.

One category of therapeutic proteins are those which in patients are not expressed, or are not expressed in a functional form, in patients due to a genetic defect. Thus administration of nucleic acid sequences encoding the missing protein can, potentially, exert a therapeutic effect. Examples of such polypeptides include CFTR, insulin, adenosine deaminase, various growth factors and blood clotting factors and so on. More

particularly of interest in the present invention are therapeutic polypeptides which are immunogenic when expressed in a human patient because they are not normal human proteins. Examples of such immunogenic polypeptides include proteins from other sources (e.g. from plants, animals, fungi, bacteria, yeasts and the like), and chimeric polypeptides which comprise portions of proteins from non-human sources, or even chimeric polypeptides which create novel fusions of human proteins or parts thereof and which are therefore immunogenic in a human subject.

Accordingly, in a particular embodiment, the invention provides a method of regulating the expression in a human or animal subject of a nucleic acid sequence encoding a polypeptide which is immunogenic in the subject; the method comprising introducing into the subject a leukocyte comprising the nucleic acid sequence encoding the immunogenic polypeptide, said sequence being operably linked to a tet operator; the leukocyte further comprising a nucleic acid sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide; and altering the concentration of tetracycline (or analogues thereof) to which the leukocyte is exposed.

Typically, the relevant nucleic acid sequences are introduced into the leukocyte *in vitro*. Numerous methods of introducing nucleic acid sequences into eukaryotic cells are known, including transfection, transduction, electroporation, cell fusion and the like. Any of these methods may be used in the present invention, and may generally be referred to as transformation.

Similarly, methods of introducing the transformed leukocyte into the subject are well known. Conveniently this is done by infusion or injection into the subject's bloodstream. It will be appreciated that introduction of foreign leukocytes into a subject is likely to create an immune response against foreign antigens on the leukocyte, so generally the leukocytes will be tissue-matched with the recipient subject. Most conveniently, the leukocytes will be autologous leukocytes originally obtained from the subject (e.g. from peripheral blood, or from bone marrow), transformed *in vitro* with the relevant nucleic acid sequences, and then re-introduced into the subject. Typically, the *in vitro* stages of the method will generally comprise a selection process to select those leukocytes

successfully transformed, and a growth stage, to increase the numbers of transformed leukocytes. Methods of selection and growth of leukocytes are well known to those skilled in the art and form no part of the present invention.

Generally, where the method involves regulating the expression of a potentially immunogenic therapeutic polypeptide, it will be desirable to cause a delay in expression of the polypeptide after the leukocyte is introduced into the subject, for reasons explained in greater detail below. Accordingly, the leukocyte is generally transferred from conditions *in vitro* in which expression of the immunogenic polypeptide is fully repressed, to conditions *in vivo* in the subject in which the expression of the immunogenic polypeptide is no longer down-regulated. However, the invention is such that it takes a significant period (typically 2 to 10 days, preferably 4 days or longer) for the leukocyte to move from the fully-repressed state to the fully-expressed state.

The leukocyte may be in a state in which the immunogenic polypeptide is fully repressed *in vitro* by exposure to appropriate (non-toxic) concentrations of tetracycline or an analogue thereof, and when introduced into a tetracycline-free subject eventually enters a state in which the immunogenic polypeptide is fully expressed. Alternatively, as described above, because of the verstatility of the TRS and the variants thereof, the leukocyte may be maintained in a fully-repressed state *in vitro* in the absence of tetracycline, and then introduced into a subject receiving appropriate doses of tetracycline or an analogue thereof, so as to cause the immunogenic polypeptide to be fully expressed (after an appropriate delay).

Generally it is preferred that the presence of tetracycline inhibits expression of the immunogenic polypeptide, as subsequent removal of the cell from tetracycline exposure normally gives a longer lag phase or delay before induction of expression of the immunogenic polypeptide.

In a particular embodiment it is envisaged that the immunogenic polypeptide is one which exerts a therapeutic effect on a solid tumour in the subject. The subject is typically a

human patient, but the method of the invention is potentially applicable to any mammalian subject, such as domesticated mammals, farm animals and so on. Thus the immunogenic polypeptide may be, for example, a cytotoxic agent (e.g. an "immunotoxin" - that is, a toxic moiety fused to an immunoglobulin binding domain, or other targeting moiety having a specific binding activity), or an agent such as an immunoglobulin, antibody, bispecific antibody or any of the other known variants of antibodies (e.g. scFv). to recruit tumour-infiltrating lymphocytes (TILs) into the tumour.

In one embodiment, the TRS controls the transcription of a replicable viral genome or a viral vector (comprising the sequence encoding the immunogenic polypeptide). Suitable constructs for achieving this embodiment of the invention have been disclosed by, *inter alia*, Hofman *et al*, (1996) and Shockett *et al*, (1995) cited elsewhere. In preferred embodiments the replicable viral genome comprises substantially that of an adenovirus or a paramyxovirus (which genome may be artificially altered by conventional DNA manipulation techniques).

The leukocytes introduced into the subject (with expression of the immunogenic polypeptide fully repressed) are able to migrate through blood vessel walls and thus penetrate into a solid tumour. Efficiency of tumour attack by the leukocytes may be enhanced by causing the leukocytes to express targeting entities on their cell surface (see, for example, Eshar *et al*, 1993 Proc. Natl. Acad. Sci. USA **90**, 720; Hwu *et al*, 1993 J. Exp. Med. **178**, 361; Stancovski *et al*, 1993 J. Immunol. **151**, 6577; and Brocker *et al*, 1996 Eur. J. Immunol. **26**, 1770). Once within the tumour, the leukocytes can exert their therapeutic effect (e.g. cytotoxic action, or recruitment of macrophages and lymphocytes).

However, targeting of the introduced leukocytes to the tumour takes some time. Thus, expression of the therapeutic polypeptide during this time is preferably avoided as it may cause: (a) collateral damage to non-malignant cells and/or (b) interaction of the therapeutic protein with components of the subject's immune system (especially circulating antibodies). This latter point is particularly pertinent if repeated administrations of the leukocytes is required, as this would cause efficient induction of immune responses to the immunogenic polypeptide, which would tend to interact with the administered leukocytes

and prevent them from reaching the target tumour. These problems can be overcome by the method of the present invention, in which the therapeutic (immunogenic) polypeptide is only allowed to become expressed at high levels after a significant time delay, by which point the administered leukocytes will have penetrated the target tumour, thereby preventing interception by the immune system and minimising collateral damage to non-malignant cells.

Any solid tumour, accessible by blood-borne leukocytes, could be available for treatment by the method of the invention. The leukocyte administered to the subject is conveniently a lymphocyte (B, or more preferably, T lymphocyte), or less preferably a monocyte or macrophage. Conveniently the leukocyte also comprises a cell-surface component which targets the leukocyte to a marker expressed on the surface of the tumour cells. These may be, for example, chimeric "T body" targeting components, known to those skilled in the art (see disclosures of Eshar *et al*, Hwu *et al*, Stancovski *et al*, and Brocker *et al*, cited above).

It may be desirable to modify the components of the TRS employed in the method so as to reduce their immunogenicity (e.g. by modifying the DNA-binding protein so as to remove certain epitopes). Conveniently the DNA-binding protein will comprise a nuclear localization signal (NLS), so as to minimise the amount which might become presented to the subject's immune system. Other useful techniques are disclosed in WO 96/01313.

In a further aspect, the invention provides a leukocyte transformed with a first nucleic acid sequence encoding a heterologous polypeptide, the first nucleic acid coding sequence being operably-linked to a tetracycline-operator sequence, and a second nucleic acid sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide, such that the leukocyte expresses the heterologous polypeptide at different levels in response to exposure to different concentrations of tetracycline or analogues thereof.

The invention also provides a composition for use in a gene therapy method, comprising a plurality of leukocytes as defined above, in a physiologically acceptable diluent medium.

The invention further provides a method of making a composition for use in gene therapy, the method comprising: obtaining a sample of leukocytes from a mammalian subject; transforming the leukocytes with a first nucleic acid sequence encoding a heterologous polypeptide, the first nucleic acid coding sequence being operably-linked to a tetracycline-operator sequence, and a second nucleic acid sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide; selecting those leukocytes successfully transformed; and mixing them with a physiologically acceptable diluent medium.

It will be apparent to those skilled in the art that the first and second nucleic acid sequences may be present on separate DNA constructs, or may be present on a single DNA construct (which is generally to be preferred as making the transformation step more efficient and easier to perform). As described elsewhere, it may also be desirable for the second nucleic acid sequence to be operably linked to a tet operator sequence, for maximal regulation.

In a different aspect, the invention provides a method of making a leukocyte differentially reactive to different densities of leukocyte-stimulating molecules present on the surface of a target cell, wherein the leukocyte is activated by an interaction between the leukocyte-stimulating molecule on the target cell and a leukocyte-activating molecule present on the surface of the leukocyte, the method comprising: transforming the leukocyte with a nucleic acid sequence directing the expression of the leukocyte-activating molecule in a manner sensitive to the concentration of an exogenous agent.

In a further aspect the invention provides a leukocyte (preferably a lymphocyte) having differential reactivity to different densities of leukocyte-stimulating molecules present on the surface of a target cell, the leukocyte having been treated by the method defined above.

Additionally the invention provides a method of altering the reactivity of a leukocyte, comprising transforming the leukocyte according to the method defined immediately above; and altering the concentration of exogenous agent to which the leukocyte is exposed.

The exogenous agent may be any agent which affects the expression of the leukocyte-activating molecule, preferably in a selective, specific manner. Preferably the exogenous agent is one which can be administered to a human patient in a pharmacological manner: that is, the agent exerts an appropriate effect on the expression of the leukocyte-activating molecule at a concentration lower than that which causes any significant toxic effect on the patient. For example, the leukocyte-activating molecule is preferably expressed in a regulatable manner using a tet O sequence and a tet-sensitive transactivator, substantially as described previously. In this embodiment, the exogenous agent may be tetracycline or an analogue thereof.

Other regulatable systems which might be used to regulate the expression of the leukocyte-activating molecule are disclosed, for example by Miller & Whelan (1997 Hum. Gene Ther. 8, 803-815).

The leukocyte is preferably a lymphocyte, most preferably a T lymphocyte. For T lymphocytes (or T cells), the leukocyte-stimulating molecule is conveniently a "foreign" antigen or a tumour-associated antigen (e.g. CEA), whilst the leukocyte-activating molecule preferably comprises the intracellular (cytoplasmic) signalling domain of at least one of the chains of the T cell receptor (TCR) CD3 complex or the intracellular signalling domain of co-stimulatory molecules such as CD28, CD4 or CD8.

For example, the leukocyte may be transformed with a sequence directing the tetracycline-sensitive expression of a TCR molecule, or of a chimeric TCR molecule which comprises at least the cytoplasmic signalling domain of the TCR molecule. Where the leukocyte-activating molecule is a chimeric TCR, the transmembrane domain (necessary to anchor the molecule on the surface of the T cell) may be from the wild type TCR molecule, or may comprise a transmembrane (TM) domain from any other molecule which is present on the surface of a eukaryotic cell. Preferably the TM domain is that from a member of the immunoglobulin family of polypeptides.

The extracellular domain of the chimeric TCR molecule typically comprises a domain which has specific binding activity for the leukocyte-stimulating molecule. In preferred

embodiments the specific binding domain is from an antibody or an antigen-binding fragment thereof (such as an scFv, Fab or the like).

In particular embodiments the leukocyte-activating molecule comprises an extracellular domain having binding affinity for a leukocyte-stimulating molecule which is a tumour-associated antigen (e.g. as described by Pardoll, *Cancer. Lurr. sp. Immunol.* (1994) 6:705).

Many tumour-associated antigens are not unique to tumour cells: they are expressed at relatively high densities on the surface of tumour cells, but may also be expressed at lower density on the surface of certain non-tumour cell types in a patient. Accordingly, many therapeutic methods which attempt to target cytotoxic agents, or to direct immune responses, to tumour cells via a tumour associated antigen often result in collateral damage to non-tumour cells.

The present invention provides a method of targeting therapeutic effects specifically to cells expressing a high density of tumour associated antigen. Expression of the leukocyte-activating molecule on the surface of the leukocyte (which delivers or mediates the therapeutic effect) can be regulated, thereby altering the density of leukocyte-stimulating molecule (e.g. tumour-associated antigen) needed to reach the threshold level of interaction at which the leukocyte becomes activated.

For example, T lymphocytes can be transformed with a nucleic acid sequence directing the tetracycline-sensitive expression of a chimeric TCR molecule having specific binding activity for a tumour associated antigen. Adjustment of the amount of tetracycline (or analogue thereof) administered to the patient can be made, such that the density of tumour-associated antigen on tumour cells is sufficient to cause activation of the T cell, whilst the density of the tumour-associated antigen on non-tumour cells is too low, minimising collateral damage. The desired adjustment of tetracycline concentration in the patient can be made by trial-and-error, by analysis of clinical symptoms or markers.

Other embodiments of the invention (e.g. relating to antigen density-specific activation of B lymphocytes) will be apparent to those skilled in the art.

The invention is exemplified below by regulation of a chimeric T cell receptor (Ch TCR), said regulation being effected by the tet regulatable system (TRS).

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which:

Figures 1A and 1B are schematic representations of nucleic acid constructs referred to in the example below;

Figures 2A, 2B and 3 show representative FACS data;

Figure 4 is a graph showing expression of a chimeric polypeptide (as a percentage of expression in control cells) against time; and

Figures 5A and 5B are bar charts showing the levels of IL-2 production (in picograms/ml) by T lymphocytes exposed to various concentrations of tetracycline or the tetracycline analogue, doxycycline.

Detailed Description of an Embodiment of the Invention

Example 1

The inventors have evaluated the utility of the tetracycline-controlled transactivator system as a means to temporally regulate the expression of a surface molecule in a human T cell line. Using a vector containing both the transactivator and the expression gene unit, we were able to generate stably transfected Jurkat T cell lines in which the expression of a chimeric TCR (chTCR) molecule could be efficiently regulated. Depending on the tetracycline analogue used and its concentration, the induction of chTCR can be reversibly repressed to a greater or lesser extent. Furthermore, we have shown that fully repressed

T cells can not be activated to produce IL-2 via this chimeric receptor, indicating that reversible functional inactivation of redirected T cells is possible.

The time-course to repress gene expression to basal levels was significantly shorter than the time-course for gene expression to reach maximal levels after drug removal, and the delay in resumption of promoter activity varied considerably depending on the concentration of doxycycline (a tetracycline analogue) used for repression. The relevance of these data to current ideas on T cell mediated immunotherapy is discussed.

Materials and Methods

Reagents. The mAbs used included SPvT3b (mouse IgG2a) (11) and YTH913.12 (rat IgG2b) (Serotec Ltd., Oxford, UK) specific for human CD3 ϵ and CD28 molecules respectively. For direct staining the following FITC-conjugated antibodies were used: UCHT-1 (anti-CD3 ϵ , a mouse IgG1 Serotec; goat polyclonal antisera to mouse λ -light chain (Southern Biotechnology Associates, Inc, Birmingham AL); and goat polyclonal antisera to mouse IgG (γ -chain specific) (Sigma Chemical Co., St. Louis, MO). Bovine serum albumin (BSA) was conjugated with 4-hydroxy-5-iodo-3-nitrophenyl acetyl (NIP) (Cambridge Research Biochemicals, Northwich, UK) in a molar ratio of 10:1 (NIP₁₀-BSA) (12). Tetracycline hydrochloride (Sigma) was dissolved at a concentration of 0.5 mg/ml in culture medium. Doxycycline hydrochloride (Sigma) was dissolved in 0.02N HCl at a concentration of 1 mg/ml and further diluted in culture medium. The antibiotic solutions were freshly prepared on the day of use and diluted to the appropriate concentrations.

Vector Construction. Plasmids pUHD 15-1, containing the tTA transactivator gene transcribed from the human CMV immediate early (CMV IE) promoter/enhancer, and pUHD 10-3, containing a tTA-responsive promoter (TRP, heptamerized tetO sequences (TetO)₇ fused to a human CMV immediate early minimal promoter [PhCMV*- 1]), were kindly provided by H. Bujard (9). Plasmid pCS was constructed by removing a 1308 bp *Sal* I fragment, containing the CMV IE promoter, the multiple cloning site and the SV40 polyadenylation signal, from pCEP4 backbone (Invitrogen, San Diego, CA). A 6723 bp *Nru* I-*Cla* I digested fragment from pCS was blunt ended with Klenow (Cambio,

Cambridge, UK) and inserted into the *Xho* I site of plasmid pUHD 15-1 following the treatment of this site with Klenow and calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim GmbH, Germany). The resulting plasmid containing both the tTA and the hygromycin transcription units in opposite directions was designated pCRAZY.

A chimeric NIP-specific scFv-TCR ζ molecule was constructed as described previously (12) and cloned into the plasmid pUHD 10-3. To do this the *Hind* III site from pUHD 10-3 was removed by cleavage with *Hind* III followed by Klenow fill-in and blunt-end ligation resulting in pLAV5. To construct pLAV6, a 1342 bp *Eco*R I-*Xba* I fragment derived from the plasmid pVAC1.aNIP.TCR ζ (described in reference 12), containing a human VH1 leader sequence and a chimeric NIP-specific TCR ζ molecule, was cloned into the *Eco*R I-*Xba* I polylinker site of pLAV5. The 91 bp *Eco*R I-*Hind* III fragment containing a Rous Sarcoma virus (RSV) promoter partial sequence was removed from pLAV6 by digestion with *Eco*RI and *Hind* III, Klenow fill-in and blunt end ligation to produce plasmid pLAV7. A polylinker containing restriction sites unique to the vector construct, *Hind* III - *Bgl* II - *Eco*R V - *Cla* I, (5826: 5'-CATCGATCGAACTGATATCAGCAGATCTCAGAAGCTTAAT-3' and 5827: 5'-ATTAAGCTTCTGAGATCTGCTGATATCAGTTCGATCGATGACGT-3') was ligated into the *Ssp* I-*Aat*II site of pLAV7 resulting in pLAV8. To construct a single plasmid with both the tTA transactivator gene and the chimeric NIP-specific scFv-TCR ζ gene under the control of TRP in antisense orientation (relative to the tTA transcription unit) the plasmid pCRAZY was digested with *Xmn* I and a *Bgl* II linker (New England Biolabs, Inc., Beverly, MA) was introduced at this site. After digestion with *Bgl* II and *Hind* III a 9386 bp fragment was inserted into the *Bgl* II-*Hind* III site of pLAV8. The resulting plasmid was designated pLAV12 (Fig. 1A).

Cell Culture and Transfections. The Jurkat T cell line (clone E6-1) was maintained in RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 25 mM HEPES buffer (all from GIBCO-BRL, Gaithersburg, MD), referred to as complete medium (CM). To generate stable cell lines Jurkat cells were transfected with linearized plasmid DNA (10 μ g) by electroporation, at 250 mV and 960 μ F, as described previously (13). Transfectants were

selected in CM supplemented with 0.4 mg/ml of hygromycin B (Calbiochem, San Diego, CA). Stable cell lines were established after 3-4 weeks and analyzed by FACS for expression of the chTCR. To select a population of chTCR ζ expressing cells, stable Jurkat cells transfected with the linearized pLAV12A or pCEP4.aNIP.TCR ζ derived plasmid fragments were FACS sorted (see below). The resultant populations were cloned twice by limiting dilution and screened for protein expression by flow cytometry.

Flow Cytometry and Cell sorting. Expression of cell surface proteins was performed by standard direct immunofluorescence as described (14) using saturating amounts of FITC-conjugated antibodies. Dead cells were excluded from analysis using a combination of propidium iodide and forward light scatter. Appropriate FITC isotype-matched irrelevant Abs were used in all experiments. The samples were analysed with a FACScan® (Becton Dickinson, Mountain View, CA). A minimum of 20,000 cells was analysed for each sample. Subsequent re-analysis of data was performed using the CELLQuest software (version 1.2) (Becton Dickinson). Additionally, cells stained with FITC-conjugated goat antisera to mouse λ light chain were sorted under sterile conditions on a cell sorter (FACScalibur, Becton Dickinson).

IL-2 Release Assay. The cells were pre-incubated at a concentration of 5×10^5 /ml for 48 hours in CM in the absence or presence of tetracycline or doxycycline at the indicated concentrations. Subsequently the cells were washed, counted and stimulated (10^5 /well) in triplicate with plastic-immobilised NIP10-BSA conjugates (iNIP10-BSA) or plastic-immobilised anti-CD3 ϵ mAb (ianti-CD3) in fresh CM alone or in the presence of the drugs (12). The plates were incubated at 37°C in 5% CO₂. After 20 hours supernatants were harvested and assayed for IL-2 activity using an ELISA kit (Genzyme Diagnostics, Cambridge, MA).

Results

Design of the tet-regulatable Vector.

To determine if the expression of a chTCR could be pharmacologically regulated in T

cells, the inventors constructed the plasmids pLAV12 and pCEP4.aNIP.TCR ζ (Figure 1). Figures 1A and 1B are schematic maps of representative plasmid fragments used in the experiments showing the predicted structure after integration into the host genome: (A) pLAV12 and (B) pCEP4.aNIP.TCR ζ , respectively. The direction of transcription is indicated by arrows.

Both constructs encode a chimeric TCR molecule that has been described previously (12), and comprises the antigen combining site of the hapten-specific (NIP) mAb B1.8 (15) fused to the transmembrane and cytoplasmic regions of the human TCR ζ chain (16). pLAV12 (Fig. 1A) is a tetracycline-regulatable construct containing all the components of the TRS with the tTA gene under the control of the constitutive CMV IE promoter and the gene coding for the chTCR inserted under the control of the tTA-responsive promoter. In an attempt to reduce potential cis-regulatory enhancement of TRP activity, due to proximity to the other enhancer and promoter elements present on the vector, the tTA-response cassette was separated from the other transcriptional units by a 2500 bp fragment containing the pUC derived ColE1 origin of replication and the β -lactamase gene (Fig. 1A). In the control construct pCEP4.aNIP.TCR ζ the chTCR molecule was under the control of the constitutive CMV IE promoter (Fig. 1B).

Both constructs encode a hygromycin resistance marker gene transcribed by a constitutive promoter. To promote the stable integration of these DNA constructs in the designed configuration in transfected T cells, a linearized *Avr II-Sap I* 9975 bp DNA fragment (Fig. 1A) from plasmid pLAV12 and a linearized *Avr II-EcoRV* 7551 bp fragment (Fig. 1B) derived from plasmid pCEP4.aNIP.TCR ζ , both lacking the EBV replication origin and the EBNA-1 gene (Fig. 1), were used to transfect Jurkat cells.

tTA-dependent Expression of the Chimeric scFv Gene Construct.

Jurkat E6-1 cells were transfected with linearized fragments of pLAV12 and pCEP4.aNIP.TCR ζ . To select for higher expression of the chTCR, the stably transfected hygromycin-resistant cells were FACS sorted after staining with a FITC-labelled goat anti mouse λ -light chain antiserum. Most of the isolated pLAV12 transfectants (JLAV12S) and pCEP4.aNIP.TCR ζ transfectants (JN3S cells), expressed the chTCR although there was

considerable heterogeneity in the absolute levels of expression (Fig. 2A, 2B).

Figure 2 shows representative results confirming the regulation of the chTCR gene expression by tetracycline analogues. In Figure 2A, stable transfected uncloned JLAV12S (left hand side) and JN3S Jurkat (right hand side) cell populations were cultured for 48 hours in tetracycline-free medium (CM, upper row of panels) or in the presence of 1 $\mu\text{g/ml}$ of Tet (broken line) or Dox (solid line)(lower row of panels) and the surface expression of chTCRs was examined after staining with FITC-conjugated goat antisera to mouse λ light chain. Figure 2B shows a timecourse of inactivation of chTCR gene expression in JLAV12S cells zero hours (top left), 8 hours (top right), 12 hours (bottom left) or 24 hours (bottom right) after addition of Dox at 1 $\mu\text{g/ml}$. In both Figures 2A and 2B negative controls (FITC-conjugated goat antisera to mouse IgG) are overlaid (filled curve). The fluorescence channel number is plotted along the x axis, and the y axis represents the relative cell number.

The selected population of pLAV12 transfectants (JLAV12S) was then cloned by limiting dilution and two subclones expressing the chTCR at low (2E11) and intermediate (1F5) levels (Figure 3) were selected for further study.

Regulation of the Chimeric scFv-TCR ζ Gene Expression.

To determine whether the expression of the chTCR could be suppressed by tetracyclines, JLAV12S and JN3S cells ($5 \times 10^5/\text{ml}$) were incubated for 48 hours in the presence of 1 $\mu\text{g/ml}$ of tetracycline (Tet) or its analogue doxycycline (Dox). At this concentration most of surface chTCRs (90%) were down-regulated in JLAV12S cells, but were not affected in JN3S cells (Figure 2A). Also, surface staining with anti-CD3 ϵ mAbs demonstrated that the amount of TCR/CD3 complex remained constant in both cell populations (not shown). No changes in cell viability were observed at this concentration, assayed using trypan blue staining (data not shown).

To study the time-course of inactivation of gene expression, JLAV12S cells were analysed at different times after addition of the antibiotics at 1 $\mu\text{g/ml}$ (Fig. 2B). A slight reduction

was observed within 8 hours of exposure to the drugs and maximum repression was achieved within 24 hours, when the expression of the chTCR fell to less than 10 percent of the level observed in the absence of tetracyclines at the same time point (Figure 2B). Similar results were observed in the 1E5 and 2E11 clones where the level of chTCR was reduced to about 10% (1E5) and 20% (2E11) of its maximal expression (Figure 3). The time-course of gene repression was very similar in response to both antibiotics (Tet or Dox) in all analysed populations. It is important to note that the percentage of cells in which tetracyclines did not down-regulate the expression of chTCRs was $< 0.5\%$, not affecting the overall regulation in the whole population of JLAV12S cells (Figure 2A).

A dose-response curve of gene repression was determined using different concentrations of Tet or Dox. After 48 hours of treatment, the cells were harvested and the expression of the chTCR was studied by FACS analysis. Typical results are shown in Figure 3.

Stably transfected uncloned (JLAV12S, left hand column) and cloned (1F5, middle column; and 2E11, right hand column) Jurkat cell populations were cultured for 48 hours in the presence of different concentrations (0ng/ml top row, 0.1ng/ml second row, 1ng/ml third row, and 10ng/ml bottom row) of Tet (broken line) or Dox (solid line) and the surface expression of scFv-TCR ζ molecules was examined. Negative controls (FITC-conjugated goat antisera to mouse IgG) are overlaid (filled curve). The fluorescence channel number is plotted along the x axis, and the y axis represents the relative cell number.

Referring to Figure 3, in both clonal populations (1E5 and 2E11) the expression of chTCRs was maximally repressed at 100 pg/ml (0.1ng/ml) of Dox, with no further increment in the level of repression at higher concentrations. Partial activity of the TRP was observed in the concentration range of 1 pg/ml to 100 pg/ml of Dox (data not shown). In JLAV12S cells maximal repression was observed at Dox concentrations greater than or equal to 1 ng/ml. For tetracycline maximal repression occurred, in all the analysed cell lines, at concentrations greater than or equal to 10 ng/ml. The induction of the chTCR gene was only partially repressed at tetracycline concentrations of 100 pg/ml to 1 ng/ml.

To study the kinetics of recovery of TRP-driven gene expression after withdrawal of tetracyclines, stable transfected uncloned JLAV12S cells were cultured for 48 hours in the presence of different concentrations of Dox (1 ng/ml to 1 μ g/ml). After three washes the cells were incubated (5×10^5 /ml) in tetracycline-free CM in new plates and the surface expression of chTCRs was examined every 24-48 hours after staining with FITC-conjugated goat antisera to mouse λ light chain. The results are shown in Figure 4. Similar experiments were also performed with 1F5 cells (data not shown).

100% values correspond to the median fluorescence of control untreated cells after staining with FITC-conjugated goat antisera to mouse light chain. The values are the percentage of chimeric TCR ζ molecules expressed on tetracycline treated cells from each cell population as compared with the amount of chimeric TCR ζ molecules on control untreated cells (as 100%). Referring to Figure 4, after treating the cells with 1 μ g/ml of Dox (filled circles), recovery of chTCR expression was not apparent at 192 hours after removal of the drug and was first detected on the cell surface after 216 hours, with full recovery of expression after 288 hours. In contrast, the TRP remained repressed for only 24 hours after drug removal, when cells were pretreated with 1 μ g/ml Tet, with maximal levels of chTCR expression being reached after 72 hours (data not shown). Treating the cells with lower concentrations of Tet (not shown) or Dox (1ng/ml - open squares, 10ng/ml - filled squares, 100ng/ml - open circles) resulted in earlier recovery of the activity of the TRP.

Example 2

Functional Study

It has previously been shown that the chTCR employed in this study is able to mediate specific recognition of its cognate antigen (NIP conjugated to BSA), soluble or plastic immobilized, resulting in the production of IL-2 by the transfected T cells (12). In the current study the inventors found consistently that stimulation of chTCR expressing cells (JN3S, JLAV12S, 1F5 and 2E11) with plastic immobilized NIP₁₀-BSA conjugates induced IL-2 secretion (data not shown). The level of IL-2 production varied between different transfectant cell populations, but in general it was similar to that observed in the same cell population in response to standardised stimulation with anti-CD3 ϵ mAb immobilized in

microtiter wells (not shown).

Given that high concentrations of tetracycline have been shown to interfere with the process of T cell activation (17), the inventors studied the effects of increasing concentrations of Tet and Dox on the anti-CD3 ϵ induced IL-2 secretion of Jurkat cells. IL-2 secretion was unaffected by 100 ng/ml or lower concentrations of doxycycline but was inhibited by 25% at a doxycycline concentration of 1 μ g/ml (not shown). Tetracycline at concentrations lower than or equal to 1 μ g/ml did not influence the anti-CD3 ϵ induced IL-2 secretion, which was similar to that observed in untreated cells (not shown). These results indicate that it is possible to induce maximal TRP repression at concentrations of doxycycline more than 1000-fold lower than the threshold at which immunomodulating effects on human T cells are first manifest.

The inventors next determined whether tetracycline-mediated suppression of the chTCR could induce a reversible state of antigen unresponsiveness in the transfected Jurkat cells. JLAV12S and JN3S cells were preincubated for 48 hours in increasing concentrations of Dox and Tet and then stimulated with immobilized NIP-BSA, following which their IL-2 production was measured. In situations of full TRP repression JLAV12S cells down-regulated 90% of surface chTCRs (see Figure 3), and were completely unresponsive to stimulation with iNIP10-BSA conjugates (see Figure 5A).

Figures 5A and 5B are bar charts showing IL-2 production (in pg/ml) by transfected JLAV12S (5A) or JN3S (5B) cells stimulated with iNIP10-BSA in the absence or presence of Tet or Dox. Cells were preincubated for a 48 hours period in the absence or presence of the drugs (at the indicated concentration in ng/ml), washed and stimulated (10^5 /well) with plastic immobilised NIP10-BSA conjugates (50 μ g/ml) in fresh CM alone (solid bar) or in the presence of Tet (shaded bars) or Dox (open bars) at the indicated concentrations. One of two similar experiments is shown.

Down-regulation of about 75% of chTCRs was associated with low IL-2 production, whereas no inhibitory effect was observed when the level of chTCR expression was about 50% of that observed in absence of tetracycline (Figure 5A). These results indicate that

the number of surface chTCR molecules expressed by JLAV12S cells in situations of full TRP repression is not enough to reach the activation threshold required for optimal T cell function. In contrast, when JN3S cells were stimulated with iNIP10-BSA conjugates, there was no inhibitory effect of tetracycline and of doxycycline at a concentration of less than 1 $\mu\text{g/ml}$ (Figure 5B).

This example shows the feasibility of using regulated expression of leukocyte-activating molecules on the surface of leukocytes to render the leukocyte differentially sensitive to different densities of leukocyte-stimulating molecules (such as antigens) present on the surface of a target cell.

Discussion

The inventors have used a single vector containing all of the components of the TRS for the pharmacological regulation of a foreign gene expressed in a human T cell line. The TRS comprises the tTA gene, usually driven by a constitutive promoter, and a gene of interest immediately downstream of the tTA-responsive promoter (9). To facilitate the application of the TRS to T cells, the inventors constructed a self-contained plasmid vector encoding both components of the TRS, as well as a hygromycin selectable marker gene under the control of a constitutive promoter. The new vector overcomes the efficiency losses inherent in co-transfection with the original two-plasmid based TRS system (9) and ensures the integration of equal copy numbers of the tTA and reporter gene units in a direct cis-configuration at the same chromosome locus.

Using this stable expression system as a model for integrated gene therapy approaches the inventors have demonstrated that a scFv-TCR ζ chimeric molecule can be functionally expressed in a human T cell line, and that its expression can be pharmacologically down-modulated leading to loss of responsiveness to the targeted antigen in the genetically modified T cells. In the absence of tetracyclines the level of expression of the chTCR was comparable to that observed when the chimeric gene was driven by the strong CMV IE enhancer/promoter. Efficient tetracycline-dependent suppression of gene expression was observed in all the studied T cell transfectants. Depending on the dose and analogue employed, the expression of chTCRs could be repressed to a greater or lesser extent. This

indicates that the TRS is applicable to T cells, achieving functional levels of transactivator expression without any evident toxic squelching effects of the VP16 domain.

Variable potencies have been demonstrated for different tetracycline analogues in previous studies on the TRS with doxycycline exhibiting 100-fold greater potency than tetracycline (18). In our system maximum repression occurred at a concentration of 10 ng/ml tetracycline whereas doxycycline caused full repression of the TRP over the range of 100 pg/ml to 1 ng/ml.

The time-course for the decline in chTCR expression upon exposure to Tet or Dox was short and suggests that, like wild-type TCR ζ chains the chimeric TCR ζ chains exhibit rapid turnover (19). In this regard it is interesting to note that the turnover of ζ chains in Jurkat cells is similar to that in primary T cells (19). In contrast to the rapid suppression of gene expression, its recovery after removal of doxycycline was much slower. The delay before the commencement of recovery of chTCR gene expression varied in direct proportion to the concentration of doxycycline that was used for repression. This sustained repression and relatively slow recovery of gene expression which occurs upon removal of tetracycline analogues has been observed in other cell types and could be of interest in the development of new T cell immunotherapy approaches and new immune evasion strategies.

Expression of the chimeric TCR conferred responsiveness to NIP-BSA conjugates, as evidenced by IL-2 secretion upon exposure to the antigen. Doxycycline was shown to completely abrogate this antigen responsiveness at concentrations that had no other immunomodulating effects on human T cells, and well below the tissue concentrations that are achieved clinically when doxycycline is used to treat infection (20). Thus, these results show that the use of TRS vectors can provide the means to render reinfused gene-modified T cells unresponsive to their targeted antigen if they cause autoimmune disorders. Likewise, by treating engineered T cells with doxycycline before their administration, it should be possible to switch off expression of the transgene for a predetermined period of time, thereby limiting collateral damage to normal tissues that may express low levels of the targeted antigen.

Interestingly, some residual expression of the chTCR in Tet or Dox treated Jurkat T cells was always detectable by FACS analysis, even when suppression was sufficient to completely abrogate responsiveness to the targeted antigen. This indicates that the number of chTCR molecules expressed in the "off" state is not enough to achieve efficient T cell activation (21), even with the experimental system used, where the cells were exposed to high concentrations of multivalent antigen. The ability easily to regulate the expression of TCR molecules at defined levels could be relevant to study the stoichiometry of the T cell activation process. On a therapeutic level, this points to the possibility of rendering T cells differentially sensitive to different surface densities of the antigen on target cell membranes.

Complete suppression of transgene expression was not fully achieved in the current study. The characteristics of the employed vector and/or cell type-specific factors may account for the level of transcription observed in the uninduced state (22, 23). In addition, the potential immunogenicity of the tTA protein must be taken into consideration. In the vector that we have employed the transactivator is driven by a constitutive promoter ensuring consistent expression independently of the presence of tetracycline, and may therefore possibly elicit an immune response against the genetically modified T cells, even in the repressed state. However, this is not the case for a newer generation of enhancerless tetracycline-responsive vectors, in which tetracycline prevents the tTA protein from binding to the TRP, thereby suppressing its own expression as well as the expression of the reporter gene by way of an autoregulatory circuit (18, 24). This arrangement gives enhanced suppression of transgene expression (to negligible levels in preliminary experiments and ensures that the abundance of the tTA protein is greatly reduced in the fully repressed state (24).

In summary we have demonstrated the use of a single tetracycline-responsive vector to achieve tetracycline-suppressible expression of a chimeric TCR gene in Jurkat T cells and we have furthermore shown that this provides a convenient method for the pharmacological regulation of the responsiveness of the engineered T cells to their targeted antigen.

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CLAIMS

1. A method of regulating the expression of a nucleic acid sequence encoding a heterologous polypeptide in a leukocyte, comprising introducing into the leukocyte the nucleic acid coding sequence operably-linked to a tetracycline-operator sequence, and a sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide; and altering the concentration of tetracycline (or analogues thereof) to which the leukocyte is exposed, so as to regulate expression of the coding sequence.
2. A method of regulating the expression in a human or animal subject of a nucleic acid sequence encoding a polypeptide which is immunogenic in the subject; the method comprising introducing into the subject a leukocyte comprising the nucleic acid sequence encoding the immunogenic polypeptide, said sequence being operably linked to a tet operator; the leukocyte further comprising a nucleic acid sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide; and altering the concentration of tetracycline (or analogues thereof) to which the leukocyte is exposed.
3. A leukocyte transformed with a first nucleic acid sequence encoding a heterologous polypeptide, the first nucleic acid coding sequence being operably-linked to a tetracycline-operator sequence, and a second nucleic acid sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide, such that the leukocyte expresses the heterologous polypeptide at different levels in response to exposure to different concentrations of tetracycline or analogues thereof.
4. A composition for use in a gene therapy method, comprising a plurality of leukocytes in accordance with claim 3, in a physiologically acceptable diluent medium.
5. A method of making a composition for use in gene therapy, the method comprising: obtaining a sample of leukocytes from a mammalian subject; transforming the leukocytes with a first nucleic acid sequence encoding a heterologous polypeptide, the first nucleic acid coding sequence being operably-linked to a tetracycline-operator sequence, and a

second nucleic acid sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide; selecting those leukocytes successfully transformed; and mixing them with a physiologically acceptable diluent medium.

6. A method of making a leukocyte differentially reactive to different densities of leukocyte-stimulating molecules present on the surface of a target cell, wherein the leukocyte is activated by an interaction between the leukocyte-stimulating molecule on the target cell and a leukocyte-activating molecule present on the surface of the leukocyte, the method comprising: transforming the leukocyte with a nucleic acid sequence directing the expression of the leukocyte-activating molecule in a manner sensitive to the concentration of an exogenous agent.

ABSTRACTTitle : Improvements in or Relating to Expression of Immunogenic Substances

Disclosed is a method of regulating the expression of a nucleic acid sequence encoding a heterologous polypeptide in a leukocyte, comprising introducing into the leukocyte the nucleic acid coding sequence operably-linked to a tetracycline-operator sequence, and a sequence encoding a tetracycline-sensitive DNA-binding expression-regulating polypeptide; and altering the concentration of tetracycline (or analogues thereof) to which the leukocyte is exposed, so as to regulate expression of the coding sequence.

1/5

Fig. 1A

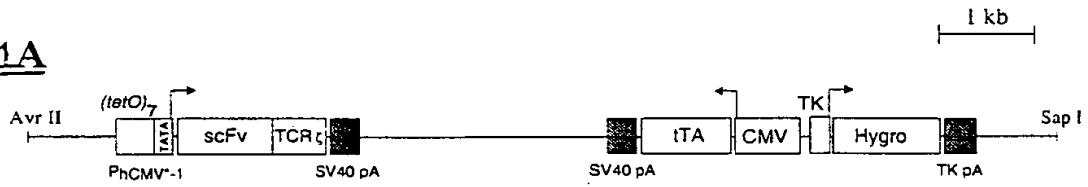


Fig. 1B

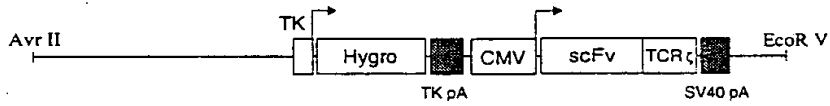


Fig. 2A

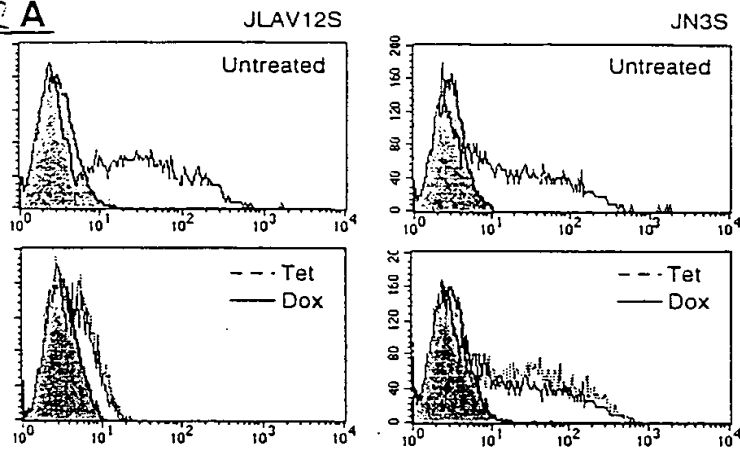
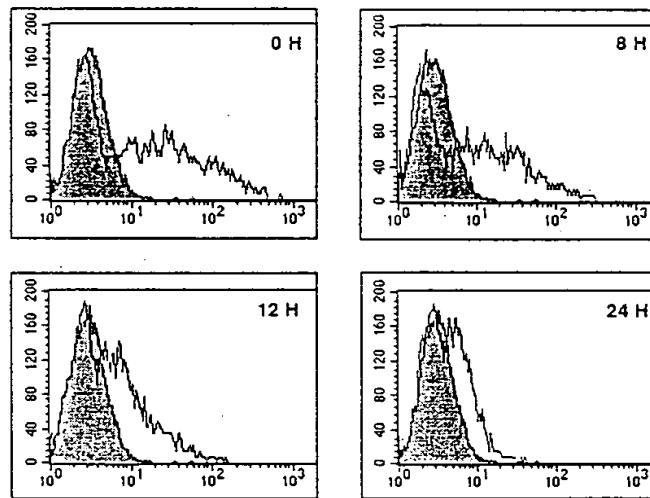


Fig. 2B



LOG. FLUORESCENCE INTENSITY

Fig. 3

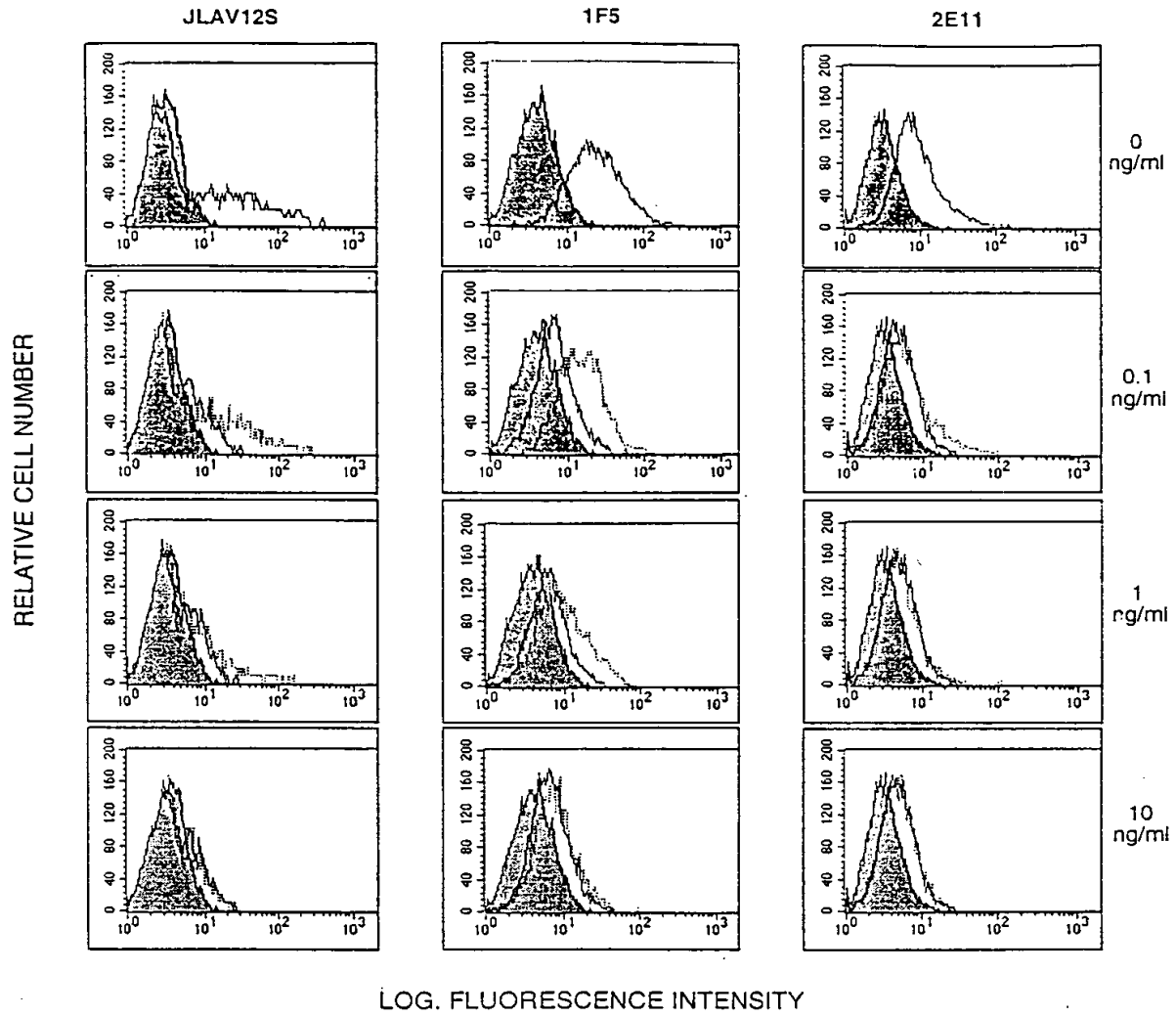


Fig. 4